MICROBIAL MATS

Physiological Ecology of Benthic Microbial Communities

Editors
YEHUDA COHEN and EUGENE ROSENBERG

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Chapter 20

Adhesion and Desorption during the Growth of *Acinetobacter calcoaceticus* on Hydrocarbons

Eugene Rosenberg, Mel Rosenberg, Yuval Shoham, Nachum Kaplan, and Nechemia Sar

INTRODUCTION

The importance of cell adhesion in the growth physiology of many bacterial species is well documented (see, e.g., references 5, 17, 36, and 40). In cases in which the carbon or energy source is a water-insoluble material such as chitin or cellulose, cell adhesion to the substrate can facilitate growth (1, 18, 38), but cell contact is not an absolute requirement, because extracellular enzymes can degrade the polymers into water-soluble substrates. However, the growth of microorganisms on hydrocarbons presents a special problem, since not only are the hydrocarbons immiscible with water, but also their breakdown cannot occur extracellularly. The final step in aromatic (8) or aliphatic hydrocarbon degradation is the introduction of molecular oxygen into the molecules by membrane-associated enzymes (6).

It has been shown that to facilitate the hydrocarbon-cell interactions, the petroleum-degrading organism *Acinetobacter calcoaceticus* RAG-1 (i) adheres avidly to hydrocarbons and other surfaces possessing hydrophobic properties (27) and (ii) produces a potent extracellular emulsifying agent (25). The purified emulsifying agent, referred to as emulsan, is an anionic heteropolysaccharide (42) containing fatty acid side chains (3), with an average molecular weight of 9.9×10^5 and an axial ratio of 60. The remarkable surface properties of emulsan depend on both its amphipathic nature (41) and its high molecular weight (37).

Production of emulsifying agents is widespread in *A. calcoaceticus* strains (32). In strain BD4, emulsification requires the presence of both a rhamnose-containing polysaccharide and a protein (15). The chemical structure of the polysaccharide has been elucidated (14), and the protein has been partially purified (15). The diversity of mi-

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crobial surfactants has recently been reviewed (21).

By using information obtained from studies with strains RAG-1 and BD4, an attempt will be made in this chapter to derive a dynamic model for the growth of *A. calcoaceticus* on hydrocarbons. The requirement of adhesion for growth on hydrocarbons will be extended to other heterotrophic bacteria in their natural environments.

BACTERIAL STRAINS AND GROWTH CONDITIONS

A. calcoaceticus RAG-1 (ATCC 31012) was originally isolated from the Mediterranean coast after enrichment on crude oil (20). The nonadherent mutant MR-481 was derived from RAG-1 (29). The partial adhesion revertant strain RV23-4 was derived from MR-481 by selection for growth on hexadecane (29). Strain TR3 is an emulsan-deficient mutant of RAG-1 (19). *A. calcoaceticus* BD4, originally isolated by Taylor and Juni (39), was provided by K. Bryn. Strain BD413, a minicapsulated (12) tryptophan auxotroph (11, 34) derived from BD4, was a gift from E. Juni. Strain BD4-R7 is a capsule-deficient, bacteriophage-resistant mutant of BD4 (15).

Growth experiments were performed at 30°C either in 250-ml flasks containing 50 ml of medium or in 14-mm-diameter Klett tubes containing 4.2 ml of medium. The flasks were incubated in a model G-53 Gyrotory shaker (New Brunswick Scientific Co., Inc., Edison, N.J.) at 150 rpm, and the tubes were incubated in a New Brunswick model G-76 Gyrotory water bath shaker at 330 rpm. Turbidity was monitored with a Klett-Summerson colorimeter fitted with a green filter.

Brain heart infusion medium was a product of Difco Laboratories, Detroit, Mich. Ac medium consisted of 0.2% sodium acetate in PUM buffer (22.2 g of $K_2HPO_4 \cdot 3H_2O$, 7.26 g of KH_2PO_4, 1.8 g of urea, 0.2 g of $MgSO_4 \cdot 7H_2O$, and distilled water to 1,000 ml; final pH, 7.1). Hx medium consisted of PUM buffer supplemented with hexadecane (olefin-free, 99% purity; Fluka AG, Buchs SG, Switzerland). GM medium contained (per liter of deionized water) 5.0 g of glucose, 9.17 g of $K_2HPO_4 \cdot 3H_2O$, 3.0 g of KH_2PO_4, 4.0 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 100-μg/ml tryptophan (final pH, 7.0). EM medium contained (per liter of deionized water) 22.2 g of $K_2HPO_4 \cdot 3H_2O$, 7.26 g of KH_2PO_4, 4.0 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 5.0 ml of ethanol. Solid media were prepared by the addition of 2% agar (Difco) to liquid media.

MEASUREMENT OF BACTERIAL HYDROPHOBICITY

Techniques

BATH Method

The bacterial adhesion to hydrocarbon (BATH) technique for measuring hydrophobicity was described previously (27, 29). Cells were harvested by centrifugation, washed twice, and suspended in 1.2 ml of PUM buffer to an initial A_{400} of ca. 1.5 (model 240 spectrophotometer; Gilford Instrument Laboratories, Inc., Oberlin, Ohio; light path, 1 cm). Various volumes of *n*-hexadecane were added, and after 10 min of incubation at 30°C the contents of each tube were mixed uniformly in a Vortex mixer (The Vortex Manufacturing Co., Cleveland, Ohio) for 120 s. After allowing 15 min for the phases to separate, we measured the A_{400} of the lower aqueous phase and compared it with that of the bacterial suspension before the mixing procedure. The fraction of adherent cells was taken as the percent decrease in A_{400} of the aqueous phase after mixing and phase separation, as compared with that of the original suspension.

DOS Method

In the direction-of-spreading (DOS) method (31, 33), a small drop of water (5 μl) was placed at the interface between the bacterial lawn and each of the following three surfaces: (i) the indicated growth medium solidified with 2% agar, (ii) a microscope glass cover slip (20 by 20 mm; Chance Propper Ltd., Warley, England [the cover slips were washed successively with ethanol and distilled water and then dried at room temperature]), and (iii) sterile polystyrene cover slips (25-mm diameter, no. 142; Lux Scientific Corp., Newburg Park, Calif. [not treated for tissue culture]). The direction to which the water drop spread was noted. Figure 1 exemplifies the method. The scoring system is summarized in Fig. 2.

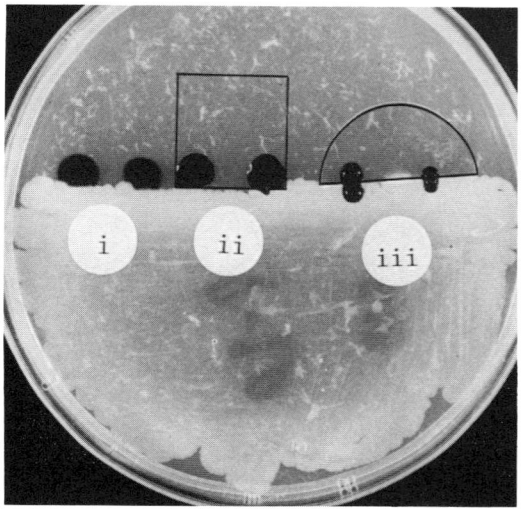

Figure 1. Measurement of the hydrophobicity of the colony surface of *A. calcoaceticus* BD4-R7 by the DOS method. After the bacterial lawn was allowed to develop for 48 h, 5-μl water drops were introduced at the border between the bacterial growth (the lower part) and one of the following surfaces: (i) 2% agar, (ii) a glass cover slip, and (iii) a polystyrene cover slip. The relative colony hydrophobicity was determined as described in the text.

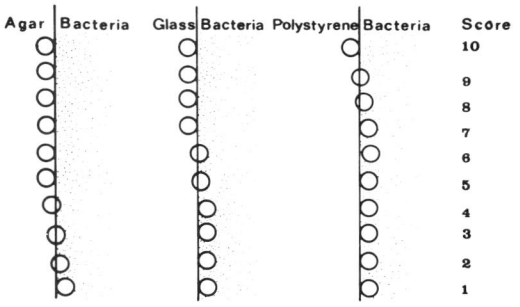

Figure 2. Scoring system for measuring the surface hydrophobicity of microbial lawns by the DOS method. A small drop of water was placed at the interface between a bacterial lawn and agar, a bacterial lawn and a glass cover slip, and a bacterial lawn and a polystyrene cover slip. For an example, see Fig. 1. The direction of spread of the drop determines the score, which is measured from 1 (the least hydrophobic bacteria) to 10 (the most hydrophobic bacteria).

Determination of BD4 and RAG-1 Capsular Polysaccharides

Culture samples were centrifuged in the cold at 12,000 × *g* for 20 min. The cell pellets were then suspended in distilled water for determination of the BD4 capsular polysaccharides by the sulfuric acid-cysteine procedure (4) with the pure capsule polysaccharide of BD4 as a standard (14), and for determination of the RAG-1 capsular polysaccharides by determining polymeric galactosamine with emulsan as the standard, as previously described (24).

MATERIALS

Emulsan, the polyanionic emulsifying agent of RAG-1, was purified from the cell-free supernatant fluid obtained from a culture grown on EM medium as described previously (25). The purified product had an emulsifying activity of 210 U/mg, an O-ester content of 0.6 μmol/mg, a reduced viscosity of 600 cm^3/g, and a residual protein content of less than 2%. The BD4 capsular polysaccharide was obtained from BD4 cells grown on GM medium. The polysaccharide was

sheared off the washed cells and purified by acetone precipitation and differential centrifugation as described previously (14). The final product contained rhamnose, glucose, mannose, and glucuronic acid in the molar ratios of 4:1:1:1.

Emulsan depolymerase was obtained from YUV-1 cells as described previously (37). The BD4 capsule-degrading enzyme was obtained from lysates of the BD4 bacteriophage ϕSL-1 (15). The crude enzyme preparation was obtained by centrifugation of the lysate at 6,000 × *g* for 20 min at 4°C, followed by precipitation of the enzyme from the supernatant fluid with ammonium sulfate (50% saturation). After standing overnight in the cold, the mixture was centrifuged at 15,000 × *g* for 20 min. The pellet was dissolved in water and dialyzed against deionized water. The resulting salt-free solutions served as the enzyme preparation.

MECHANISMS OF ADHESION

Adhesion of *A. calcoaceticus* RAG-1 to Hexadecane

Microscopic observations of samples taken during the growth of RAG-1 on hydrocarbons indicated that during the exponential phase of growth, most of the RAG-1 cells were adhering to the hydrocarbon/water interface. To study this adhesion phenomenon we developed a simple adhesion test, now referred to as the BATH test. The test is not specific for hydrocarbon-degrading bacteria, but, rather, is a measure of cell surface hydrophobicity.

RAG-1 cells showed 95 to 100% adhesion to hexadecane (Table 1). By sampling the aqueous phase and repeating the adhesion assay, it was possible to obtain a mutant of RAG-1, referred to as MR-481, which failed to adhere to 0.05 to 0.2 ml of hexadecane.

Table 1.
Adhesion of *A. calcoaceticus* Strains to Hexadecane

Strain[a] and hexadecane concn (ml)	Adhesion[b] (%)
RAG-1	
0.05	100
0.1	98
0.2	98
MR-481	
0.05	0
0.1	0
0.2	0
RV23-4	
0.05	30
0.1	40
0.2	55

[a] Cells were grown to stationary phase in brain heart infusion broth.
[b] Adhesion was measured as a function of hydrocarbon volume.

Role of Adhesion in Growth of Strain RAG-1 on Hexadecane

Strains RAG-1 and MR-481 both grew well on acetate as a carbon source, reaching approximately 200 Klett units in 15 h (Table 2). However, the nonadhering strain failed to grow on hexadecane medium for at least 30 h, whereas RAG-1 grew well after a short lag, reaching 100 Klett units in 15 h. The latter experiment was conducted with low initial cell densities (ca. 5 Klett units) and low mechanical agitation to maximize the need for adhesion in the cell-hydrocarbon interaction. When the experiment was carried out in the presence of emulsan (to increase the surface area of the hexadecane) strain MR-481 grew well. Therefore, MR-481 had the potential to grow at the expense of hexadecane once it came into close contact with the substrate.

After a few days of incubation of MR-481 in hexadecane medium, cells appeared which were capable of growth on hexadecane under these restrictive conditions. One of these mutants, referred to as RV23-4, was cloned and studied. Strain RV23-4 was a partial revertant with regard to both adhe-

Table 2.
Growth of *A. calcoaceticus* Strains in Acetate- and Hexadecane-Containing Media[a]

Strain and incubation time (h)	Turbidity (Klett units) in:	
	Ac medium	Hx medium
RAG-1		
5	55	6
10		60
15	205	110
MR-481		
5	53	5
10		4
15	200	3
RV23-4		
5	53	5
10		10
15	205	40

[a] Washed cells were inoculated into acid-washed Klett tubes (diameter, 14 mm) containing 4.2 ml of Ac medium (PUM buffer supplemented with 0.2% sodium acetate) or Hx medium (4.2 PUM buffer plus 0.5 ml of hexadecane) to give an initial turbidity of 5 Klett units.

sion to hexadecane (Table 1) and growth on hexadecane (Table 2). Studies with a number of different adhesion mutants of RAG-1 have demonstrated that adhesion is a crucial factor in the growth of RAG-1 on water-insoluble hydrocarbons in the absence of emulsification of the substrate.

Mechanism of RAG-1 Adhesion to Hydrocarbons

Henrichsen and Blom (10) described two types of fimbriae, ca. 3 and 5 nm in diameter, present on various *A. calcoaceticus* strains. The thick fimbiae were correlated with twitching motility. No physiological role was proposed for the thin fimbriae. Thick fimbriae (6.5 nm) are present on RAG-1, MR-481, and RV23-4. Thin fimbriae (3.5 nm) are the major organelles responsible for adhesion of the cells to hydrophobic surfaces (26). Washed cells containing thin fimbriae adhere avidly to hydrocarbons, including nonutilizable aromatic hydrocarbons. Nonadhering mutants, such as MR-481, lack the thin fimbriae, fail to adhere to hydrocarbons, and grow poorly on water-insoluble hydrocarbon substrates. Revertants of these mutants, such as RV23-4, regain the ability to adhere to hydrocarbons and again show the presence of the characteristic thin fimbriae (26). Additional evidence for the role of thin fimbriae in adhesion was obtained by shearing the cells, with concomitant loss of the fimbriae and cell surface hydrophobicity.

Role of Emulsans

Although emulsans were initially isolated and characterized as extracellular emulsifying agents (13, 20, 25), it is now clear that both BD4 and RAG-1 emulsans have their origin in capsular polysaccharides that are released from the cell surface during the stationary phase or under conditions of unbalanced growth. With radioactive ethanol as a carbon source, pulse-chase experiments established that the RAG-1 emulsan polysaccharide, released during chloramphenicol treatment, was present on the cell surface before addition of the chloramphenicol (30). Further evidence for a cell-associated form of emulsan came from chemical analyses of washed cells (Table 3), electron microscopy (2), and immunochemical studies (9).

The demonstration that the emulsan

Table 3.
Release of Emulsan from the RAG-1 Cell Surface

Growth stage[a]	Emulsan (% of cell dry wt)	
	Capsular	Extracellular
Exponential phase	14	0.6
Plus chloramphenicol[b]	0.5	15
Stationary phase	2.0	22

[a] Cells were grown in EM medium (see the text).
[b] Exponential-phase cells were harvested and suspended in EM medium containing 25 μg of chloramphenicol per ml; the chloramphenicol-treated cells were incubated for 4 h at 30°C.

Table 4.
Inhibitory Role of Cell-Associated Capsules in Adhesion of *A. calcoaceticus* to Hexadecane

Strain[a]	Capsular polysaccharide (% of cell dry wt)	Adhesion (%)
BD4	27	0
BD413	11	70
BD4-R7	0	97
BD4[b]	3	98
RAG-1	17	61
RAG-1[b]	3	93
TR3	4	85

[a] Strains BD43, BD4-R7, and BD413 were grown in GM medium to stationary phase. Strains RAG-1 and TR3 were grown in EM medium and harvested during exponential phase.
[b] These strains were enzymatically decapsulated. Decapsulation was carried out with BD4 phage capsule depolymerase (for BD4) (15) and emulsan depolymerase (for RAG-1) (38).

polysaccharides were cell surface polymers suggested that they might be involved in adhesion to hydrocarbons. However, the evidence (Table 4) indicated that the emulsan capsules decreased the ability of BD4 and RAG-1 to adhere to hydrocarbons (24). Removal of the emulsan capsule, either by mutation (BD4-R7 and TR3) or by enzymatic degradation, significantly enhanced cell surface hydrophobicity. During the exponential growth phase of RAG-1 on hydrocarbons, the cells were observed to be tightly bound to the oil droplets. When growth slowed, emulsan was released into the medium and an increasing percentage of the cells were found free in the medium. This increasing number of unbound RAG-1 cells at a time when the cells were becoming more hydrophobic, owing to release of capsular emulsan (Table 4), can be understood if one realizes that the surface of the emulsified oil (hydrocarbon-water) has become hydrophilic. It is known that purified emulsan inhibits the adhesion of cells to hydrophobic surfaces (22). The inhibition of adhesion can be reversed by degrading the emulsan with emulsan depolymerase.

COMPARISON OF BATH AND DOS METHODS

The hydrophobicity of colonies or surface layers of bacteria is not necessarily correlated to the hydrophobicity of the dispersed cells making up these colonies (33). The capsule-deficient BD4-R7 was very hydrophobic by both methods, whereas RAG-1 and TR3 adhered avidly to hexadecane, but showed only intermediate colony surface hydrophobicity (Table 5). Although strain BD4 failed to adhere to hexadecane, it yielded as high a surface hydrophobicity as strains RAG-1 and TR3 did (Table 5).

DYNAMICS OF GROWTH OF *A. CALCOACETICUS* ON PETROLEUM

Studies on the growth, surface properties, and production of emulsans by *A. calcoaceticus* RAG-1 and BD4 have led to a model of growth on water-insoluble hydrocarbons that comprises the following steps.

(i) Individual cells adhere to the surfaces of large oil drops. Adhesion of RAG-1

Table 5.
Comparison between Colony Hydrophobicity (DOS method) and Cell Surface Hydrophobicity (BATH method)

Strain	Hydrophobicity	
	BATH method (% adhesion)[a]	DOS method[b]
RAG-1	100	5
MR-481	25	4
TR3	97	6
BD4	3	6
BD4-R7	97	9

[a] Strains RAG-1, MR-481, and TR3 were grown on brain heart infusion agar; strains BD4 and BD4-R7 were grown on GM agar. Cells were removed from the agar medium and suspended directly in PUM buffer. Each cell suspension was washed twice by centrifugation and resuspension before adhesion to 0.2 ml of hexadecane was measured as described in the text.
[b] The hydrophobicity of each colony surface was determined by the DOS method as described in the text and illustrated in Fig. 1. The scoring system for the DOS method is shown in Fig. 2.

is mediated by the presence of hydrophobic fimbriae which are distributed over the entire surface of the bacterium.

(ii) The bacteria grow as a biofilm at the hydrocarbon/water interface, extracting water-soluble minerals, nitrogen, and phosphate from the aqueous phase and oxidizing *n*-alkanes to carboxylic acids, which provide the carbon and energy source. Growth is restricted to the hydrocarbon/water interface.

(iii) During the growth phase, the cells accumulate emulsan on their surfaces in the form of a capsule. As the encapsulated cells cover the oil droplets, they reduce the interfacial tension between the hydrocarbon and the water. This results in the emulsification of the oil and an increase in the oil-water surface area available for growth. During the growth phase, the individual oil droplets that have been colonized by the bacteria become depleted of *n*-alkanes. These droplets become enriched in nonutilizable aromatic and cyclic hydrocarbons.

(iv) When the *n*-alkane content of the colonized droplets becomes depleted, the cells become starved. The starved cells release their emulsan capsules. The extracellular emulsan attaches avidly to the oil droplet, thereby displacing the cells to the aqueous phase. Each "used" droplet is then covered with a stable monomolecular film of emulsan. The fatty acid esters and amides of emulsan are oriented into the oil phase, whereas the hydrophilic carboxylic and hydroxyl groups of the polysaccharide face the aqueous phase. The hydrophilic outer surface of the emulsan-coated oil prevents reattachment of the free bacteria. The released capsule-deficient bacteria are extremely hydrophobic and readily adhere to fresh hydrocarbon substrate.

CELL SURFACE AND COLONY HYDROPHOBICITY

The BATH test measures the adhesion of individual suspended cells to hydrocarbons. For a cell to partition to the hydrocarbon/water interface, it must contain a hydrophobic region on at least part of its outer surface. Other measures of cell surface hydrophobicity include hydrophobic interaction chromatography and binding of radiolabeled hydrophobic compounds (28).

The DOS test measures the hydrophobicity of the upper layer of cell lawns or colonies. The direction of spreading of a water drop between two surfaces is, in fact, a satisfactory definition of hydrophobicity. In principle, the forces which govern the spreading of a water drop between two surfaces are the same as those measured by the contact-angle method (7).

Comparison between cell surface and colony hydrophobicity of a number of different bacteria resulted in a wide variety of relationships. In some cases both the individual cells and the colony surface showed similar hydrophobicity values, whereas in other cases there was a large difference between cell and colony hydrophobicity. The differences between cell and colony hydrophobicity values can be explained in at least two ways. (i) The colony can be differentiated so that the cells at the surface of the colony have an intrinsically different cell surface hydrophobicity from that of the interior cells. (ii) The cells produce extracellular slime, which concentrates at the surface of the colony and thereby imparts to it a different hydrophobicity from that of the individual cells. It may be assumed that part or all of this extracellular slime can be removed from the cells during suspension and washing procedures. In this regard, it is interesting that Shapiro (35) has observed, by using scanning electron microscopy, a thin layer of extracellular surface material covering the colony. Also, during the development of *Myxococcus xanthus*, a loosely bound extracellular material is produced which can change the cell surface hydrophobicity (16).

GENERAL REQUIREMENT FOR ADHESION AND COLONY FORMATION IN THE GROWTH OF HETEROTROPHIC BACTERIA IN NATURE

Shilo has outlined the general requirements for adhesion and growth of benthic cyanobacteria (chapter 18 of this volume). The data presented here demonstrate that adhesion and colony formation are also requirements for the growth of *A. calcoaceticus* RAG-1 on hydrocarbon substrates. However, it can be argued that hydrocarbons, although released into water and onto land by natural oil seeps, are unusual substrates from which to generalize on the growth patterns of bacteria. What, then, are the typical growth substrates for heterotrophic bacteria in natural water and soils?

The most abundant sources of organic material for microorganisms are probably particulate matter, complex polysaccharides, and structural proteins. For bacteria to utilize these carbon sources, the cells must produce a battery of extracellular enzymes. Consider, for example, an individual bacterium dispersed in a natural body of water containing a low concentration of dispersed particulate matter. The cell secretes its extracellular enzymes, which diffuse into the water. The overall concentration of enzymes produced from a single cell is very low and would not produce a high enough concentration of permeable substrate to allow growth. If, on the other hand, the cell was adhering to the substrate, then the local concentration of enzyme and resulting permeable substrate would be high enough to initiate growth. Furthermore, the resulting colony of biofilm would produce a high local concentration of enzyme that would ensure efficient continued growth. This type of cooperative growth kinetics has been demonstrated in the model case of *M. xanthus* growing on casein (23).

The above arguments lead to the conclusion that bacteria in nature typically adhere to surfaces and form colonies, mats, and biofilms.

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Chapter 23

Microbial Films in the Mouth: Some Ecologically Relevant Observations

Mel Rosenberg

INTRODUCTION

One need not travel great distances to investigate an exciting, naturally occurring macroscopic biofilm—a look in the mirror is quite sufficient. Dental plaque, the macroscopic accumulation of bacteria on tooth surfaces, is a convenient, close-at-hand biofilm of great importance to humans. Dental caries and periodontal disease, the two most widespread diseases of modern humans, are linked to the activity of microorganisms on tooth surfaces (7, 12, 17). The soft tissues in the mouth are also colonized by biofilms, which are generally sparser than dental plaque, presumably owing to the shedding (desquamation) of the outermost epithelial cell layers. Finally, dental restorations, dentures, and devices are similarly prone to rapid biofouling in the oral cavity.

Microbial accumulations in the oral cavity appear to share several characteristics with biofilms in the open environment, as follows. (i) Similar to microbial films which form on surfaces in contact with water flow (pipes, rocks and sediments, submerged aquatic life, etc.), oral microorganisms must adhere to remain in their ecological niche and to survive. (ii) Similar to many environmental biofilms, bacterial accumulation on oral surfaces entails a complex physical and physiological coexistence involving a wide variety of bacterial species (7, 12). (iii) Maturing dental plaque results in a succession of anaerobic and acid-tolerant populations in close proximity to the tooth surface (7, 12). (iv) With prolonged passage of time, dental plaque accumulations become increasingly recalcitrant and calcified (dental calculus) and may be removed only by strong mechanical action (e.g., sonic oscillation). (v) Analogous to the chemical and structural changes in the underlying substratum as a result of many biofilms (e.g., corrosion of metals), tooth decay (dental caries) results from destruction of the protective enamel layer, primarily by acid accumulation (7, 12, 17). Finally, similar to the odorous, volatile gases produced by microbial mats and other biofilms, volatile gas elaborated by microbial accumulations in the mouth itself is the primary cause of bad breath (halitosis) (33, 36).

In this chapter I discuss some "ecological" aspects of oral biofilms which have attracted the interest of investigators in this

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and other laboratories: (i) adhesion, colonization, and desorption of oral bacteria; (ii) sampling and localization of adherent microbial populations on tooth surfaces; and (iii) measurement and monitoring of volatile, sulfur-containing gases in the oral cavity.

ADHESION, COLONIZATION, AND DESORPTION

Adhesion and Colonization

Cleaned surfaces inserted into the oral cavity or, alternatively, immersed in saliva become conditioned with a coat of salivary components prior to microbial adhesion (7, 12). This layer, termed the acquired pellicle, may differ to some extent in composition, distribution, or juxtaposition of adsorbed salivary molecules, depending on the substratum characteristics; it is likely, however, that many salivary components adsorb to a wide variety of oral surfaces (12).

Researchers studying the oral cavity are in general agreement that microbial adhesion is a crucial initial process in the colonization of oral surfaces; however, the actual mechanisms which promote oral adhesion have been a subject of debate for years (7, 12, 15–17, 30, 31). Similarly, the subsequent role of adhesion in biofilm buildup on oral surfaces is unclear: dental plaque accumulation appears to be due primarily to outgrowth of already attached bacteria, rather than to adsorption of free cells from saliva (12).

One striking observation of oral biofilms is the heterogeneity of their microbial composition. For example, high levels of *Streptococcus salivarius* are found attached to the tongue dorsum and buccal surfaces, but very low levels are observed in dental plaque. *Streptococcus mutans*, on the other hand, colonizes tooth surfaces but not soft oral tissues and is thus absent from mouths that are free of teeth or artificial hard surfaces (7). Striking differences in microbiota are also observed microscopically: whereas coccal forms are predominant on buccal epithelial cells, noncoccal forms, such as rods, filamentous bacteria, and fusiform bacteria, constitute a large proportion of dental plaque (7). Gibbons et al. have postulated that these differences reflect specific adhesion mechanisms (7, 10). Thus, according to this hypothesis, oral bacteria adhere with high affinity to surfaces on which they are able to proliferate, but adhere poorly to surfaces which they do not colonize.

During recent years, there has been an increasing number of reports that hydrophobic interactions play a significant role in bacterial adhesion in the open environment. In 1982, Weiss et al. (40) and Nesbitt et al. (21) reported findings suggesting that bacterial hydrophobicity may be important for oral adhesion as well. During the past 5 years, this possibility has been explored extensively in several laboratories.

The following lines of evidence support a major role for bacterial hydrophobicity in oral adhesion. (i) Many oral bacteria exhibit pronounced cell surface hydrophobicity, as measured by adhesion to hydrocarbons; they include fresh oral isolates (40), caries-associated and periodontal disease-associated laboratory strains (5, 8, 14, 19, 21, 23, 42), dental plaque itself (29), and bacteria obtained directly from saliva (15, 16). (ii) In several cases, clear-cut correlations were observed between adhesion to saliva-coated hydroxyapatite (SHA) beads (an in vitro tooth surface model system) and cell surface hydrophobicity in various oral strains (8, 19, 23) and following various treatments (11, 22); moreover, adhesion to SHA can be blocked by hydrophobic-bond-breaking agents, e.g., thiocyanate and tetramethyl urea (21, 38). (iii) Nonhydrophobic mutants of hydrophobic strains are deficient in adhering to tooth surfaces (39) or SHA (6, 9, 19). (iv) In an in vivo study, Svanberg et al. showed that a hydrophobic fresh isolate of *S. mutans* is retained in the human oral cavity, whereas its nonhydrophobic variant is not

(34). (v) Emulsan, a bacterial amphiphile which blocks adhesion to hydrophobic surfaces, similarly inhibits the adhesion of oral and nonoral strains to buccal epithelial cells (26).

If hydrophobic interactions constitute a major mechanism for oral adhesion, one would expect that adhesion to various oral surfaces would not be as specific as previously suggested (31). Indeed, although filamentous plaque bacteria do not adhere significantly to buccal epithelial cells in situ, adhesion readily takes place in a saliva-free in vitro system (35). Moreover, this binding is inhibited by amphipathic agents and saliva (M. Rosenberg and S. Guendelman, unpublished data). Thus, the heterogeneity of microbial populations on various tooth surfaces may not be a reflection of their adhesion capabilities, but rather their relative ability to colonize and proliferate on various oral substrata (31).

Several recent investigations have challenged the role of hydrophobicity in oral adhesion. Van der Mei et al. (37), using six different methods for studying hydrophobicity, were unable to demonstrate a clear correlation between hydrophobicity and adhesion to SHA. Similarly, Wyatt et al. (43) reported that although all strains of *Streptococcus sanguis* tested adhered to hexadecane, large differences in adhesion to SHA were observed. Knox et al. (13), using continuous-culture-grown cells, found no correlation between adhesion to hexadecane and to SHA. Clark et al. (2) found a strong correlation between the relative hydrophobicity of 42 oral *Actinomyces* strains and adhesion to SHA, but were unable to observe inhibition of adhesion of SHA by the surfactant Tween 80.

These apparent disagreements can be resolved only through further research and improved methodology. It is likely that SHA may be an interesting model system with which to study adhesion, but that it does not clearly mimic the tooth surface itself; moreover, this technique is used with various significant alterations in different laboratories. Similarly, improved bacterial hydrophobicity tests could be of great assistance (30). In any case, it is worth emphasizing that the current hydrophobicity tests do not measure the same characteristics (32) and that no single test is definitive in measuring these properties (4, 20). This point is well illustrated by the recent reports of Weerkamp et al. (38) and van der Mei et al. (37). These investigators did find overall correlations between the adhesion of wild-type *S. salivarius* and various mutants to hexadecane and SHA. One mutant (HB-7), however, was exceptional in that it adhered to hexadecane but not to SHA. The investigators inferred from this observation that adhesion to SHA cannot be accounted for by bacterial hydrophobicity. However, this mutant adheres poorly to the hydrophobic plastic polymethylmethacrylate (37). Opposite observations have been made with *Acinetobacter calcoaceticus* BD4, which adheres to polystyrene but not hexadecane (27, 30). Finally, it should be emphasized that adhesion characteristics, including cell surface hydrophobicity, may be lost upon subculturing (41).

To summarize, the extent to which hydrophobic interactions mediate adhesion within the oral cavity remains unresolved at present. Some investigators have proposed a major role for cell surface hydrophobicity in mediating oral adhesion (16, 23, 31, 38); others have urged fellow researchers to desist this line of study altogether (25); still others have proposed that hydrophobic interactions act in concert with additional mechanisms (3, 5, 21, 38). Indeed, the findings that the lactose-sensitive coaggregation of oral bacteria may have hydrophobic components (18) suggest that a precise line between stereochemically specific and nonspecific adhesion mechanisms may be hard to draw. Finally, bacteria may adhere by one mechanism to other bacteria, which are themselves bound by another mechanism to the tooth surface (1).

Bacterial Desorption from Oral Surfaces

Among the treatments to eliminate or reduce microbial buildup on surfaces, desorption of attached cells is a logical choice, since inhibition of the initial adhesion processes is often unfeasible. Desorption itself is a more complicated process than inhibition of adhesion, since a bacterium may adhere via a multitude of molecular interactions, which are difficult to break simultaneously; moreover, bacterial elaboration of extracellular polysaccharides may help cement already adherent cells.

The observation that a large proportion of oral bacteria adhere to oil droplets prompted us to propose swishing with an oil-water rinse as a technique of desorbing adsorbed bacteria from oral surfaces (E. Weiss, M. Rosenberg, and H. Judes, U.S. patent 4,525,342, June, 1985). In such a case, the actual adhesion mechanisms are irrelevant as long as the affinity of the cells for the oil droplets can overcome the adhesive forces. In addition to their ability to desorb oral bacteria, oils have been shown to be capable of desorbing bacteria from polystyrene (28) and fish skin (32). During our studies, the possibility arose that oil droplets could also bind amphipathic odor-causing molecules (Weiss et al., patent) and perhaps serve to reduce halitosis levels. This, in turn, prompted us to look for a simple means of studying halitosis, which is discussed later on.

SAMPLING OF TOOTH SURFACES FOR SPECIFIC ADHERENT MICROORGANISMS

Several convenient methods (Rodac plates, contact dip slides, adhesive tape, etc.) exist for sampling microorganisms from smooth surfaces. However, the localization and sampling of specific microorganisms adhering to rough surfaces, e.g., tooth surfaces, present a problem. Recent studies have shown that early dental caries formation is linked to the presence of *S. mutans* (17). The levels of desorbed *S. mutans* cells in saliva are currently used to assess dental caries levels and susceptibility.

We were interested in trying to develop a simple technique to pinpoint the growth of *S. mutans* on the tooth surfaces, rather than measure the levels of desorbed cells in saliva. To this end, we obtained impressions of the tooth surface by using a tacky, malleable material. Cells not adhering to the impression material were washed off in tap water. Liquid agar-containing media were cooled to 45°C and poured over the impressions, but growth was difficult to discern. To simplify the process, we decided to attempt to incubate the impression in a liquid medium. To confine outgrowth to the solid/liquid interface, the major carbon and energy source (sucrose) was impregnated in the impression material but was absent from the liquid medium. By incorporating selective, chromogenic components in the liquid outgrowth medium, specific growth of *S. mutans* on the impression surfaces was clearly observable (31a). This technique may have clinical and ecological implications in mapping the presence of this microorganism on tooth surfaces and, perhaps, in providing an indication of incipient caries formation. Minor modifications of the method may also facilitate sampling and concomitant localization of adherent populations on rough surfaces in the environment.

HALITOSIS

As stated above, the production of foul oral odors is analogous to putrid-gas elaboration by microbial mats and biofilms in the open environment. However, halitosis is the cause of substantial human discomfort; unfortunately, relatively little research has been directed toward this problem, which is said to affect the majority of adults in the Western world (36). About 85 to 90% of

halitosis cases are reported to be oral in origin: the direct consequence of metabolic activity of bacterial accumulations on various oral surfaces (36). Volatile sulfur-containing gases such as hydrogen sulfide, methyl and ethyl mercaptan, and dimethyl sulfide are a major factor in oral malodor. Most halitosis cases can be alleviated by proper oral hygiene and dental techniques (36). We have recently suggested that monitoring the levels of halitosis may aid the dental practitioner in treating this common ailment. Previously, halitosis measurements have been made by gas chromatography (33, 36), which is too complicated and time-consuming for routine clinical testing, or by an organoleptic panel of volunteers, which is similarly unsuitable. We have recently tested a portable, simple-to-use industrial sulfide monitor, which can measure sulfide concentrations below 0.1 mg/liter; initial results suggest that such testing can provide relevant quantitative data (24). The use of such instruments for field measurement of volatile sulfur produced by microbial mats and other biofilms may also be of interest.

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